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TITLE: Follistatin: A Potential Anabolic Treatment for Re-Innervated Muscle

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## 1. Introduction:

Functional recovery following major peripheral nerve injuries is often suboptimal despite adherence to well accepted nerve repair principles. Though a multifaceted problem, the poor muscle functional recovery often seen following nerve regeneration is in large part due to the progressive catabolic process affecting muscle fibers called “denervation atrophy.” While many researchers have approached this issue by attempting to improve axonal regeneration speed, efficiency, and accuracy (and thereby limiting the degeneration of the muscle), we have sought treatment options aimed at maximizing the potential of the muscle fibers that were able to achieve reinnervation. After experimenting with anabolic steroids (nandrolone), we determined that a more potent but safer anabolic agent would be a better option. Follistatin is a glycoprotein that both blocks the muscle inhibiting peptide myostatin and possesses remarkable independent muscle stimulating properties as well. We hypothesized that the administration of recombinant follistatin delivered to rodent muscles subjected to prolonged but temporary denervation periods (of either 3 or 6 months) would improve final muscle recovery and function. Most published studies have delivered the follistatin as recombinant DNA though some successful administration of recombinant protein has been demonstrated as well leading us to form two wings for our study—one exploring recombinant DNA administration and one exploring protein administration.

## 2. Keywords:

Denervation atrophy, anabolic, follistatin, nerve injury, nerve repair, muscle

## 3. Accomplishments:

*Major goals:*

- **Specific Aim 1: Utilize an established rodent model of denervation atrophy**
- Regulatory Review and Approval Process- *complete*
- Testing the Protein Stability- *complete*
- **Pilot Study** (N=15; Follistatin recombinant DNA, Protein, and Alzet Pump Control Groups). Each group has 5 animals.- in progress: control group complete, 2/5 protein pump complete (other three will be complete next week), and recombinant DNA pilot initiated (expect results in 4 weeks) (*75% complete*)
- Denervation of hind limb muscles (3 months) Six groups (N=12; total of 144 rodents) will be divided into control (sham surgery, sham treatment), sham surgery, sham treatment, and experimental groups (denervation surgery + treatment). Experimental and sham treatment groups will undergo left tibial nerve transection to denervate left gastrocnemius muscle. Control and sham surgery groups will undergo exposure of the nerve without transection.- *pending*
- Re-innervation of hind limb muscles. (3 months) Denervation will be reversed by repairing the transected tibial nerve using graft obtained from contralateral tibial nerve. Control rats will still undergo harvest of graft without repair.- *pending*
- **Specific Aim 2: Treat re-innervated muscle with Follistatin:**
- Synthesis of Recombinant Follistatin DNA/Protein (4 months) Recombinant follistatin isoform (FS-288) and recombinant follistatin DNA (FS-288) synthesized by the Virginia Commonwealth University Biological

Macromolecule Core facility. Recombinant follistatin DNA will be packaged in adeno-associated virus (AAV) vectors. Will be prepared as needed for treatment. - *pending* (Vector BioLabs now producing Recombinant DNA and AAV; enough material provided to complete pilot study; if pilot study works they would be able to provide enough material to continue with study in 2-3 weeks. BioVision now providing protein which they have stockpiled. They have provided enough material for pilot study. If pilot successful (which initial data is supportive), they will be able to provide material in 2-3 days).

- Treatment of re-innervated hind limb muscles (3 months) All rats will undergo either injection of recombinant follistatin DNA packaged in AAV (into gastrocnemius muscle) or implantation of drug delivery reservoir (with either carrier or recombinant follistatin protein + carrier). - *pending*
- **Specific Aim 3: Determine treatment effects utilizing strength testing, muscle morphology, electrophysiology nerve testing**
- Testing of muscle recovery/nerve regeneration (3 months) All rats to undergo muscle morphology measurements, nerve conduction, and force generation studies of tibial nerve and gastrocnemius muscle. - *pending*
- Immunohistology staining and histology of muscle (3 months) Fiber type analysis and satellite cell quantification to be determined for all specimens. - *pending*
- Measurement of Follistatin levels in muscle (3 months) immunoassay - *pending*
- **Specific Aim 4: Histology (of nerve and muscle), Manuscript preparation, Presentation**
- Histology of muscle/nerve (3 months) Cross sections of muscle specimens will be stained and fiber size, axon numbers, and myelination measured. - *pending*
- Data Analysis (3 months) - *pending*
- Manuscript Preparation (3 months) – *pending*

In consultation with DOD scientific officer and in response to reviewer critiques, the decision was made to perform a pilot study to ensure expected anabolic result following administration of Recombinant DNA or Recombinant protein. The main experiment is therefore on hold pending completion of the pilot study.

- Regulatory review process complete and IACUC and ACURO communications reflecting changes to protocol are current and up to date.
- VCU Biological Macromolecular core facility accomplished the following:
  - Constructed AAV (adeno-associated virus) vector with Recombinant FS-288 DNA
    - Unable to produce in adequate quantities for full scale experimental model
  - Constructed AV (adeno virus) vector with Recombinant FS-288 DNA

- AV with Recombinant FS-288 administered to mouse muscle cells in vitro stimulated differentiation (Figure 1).

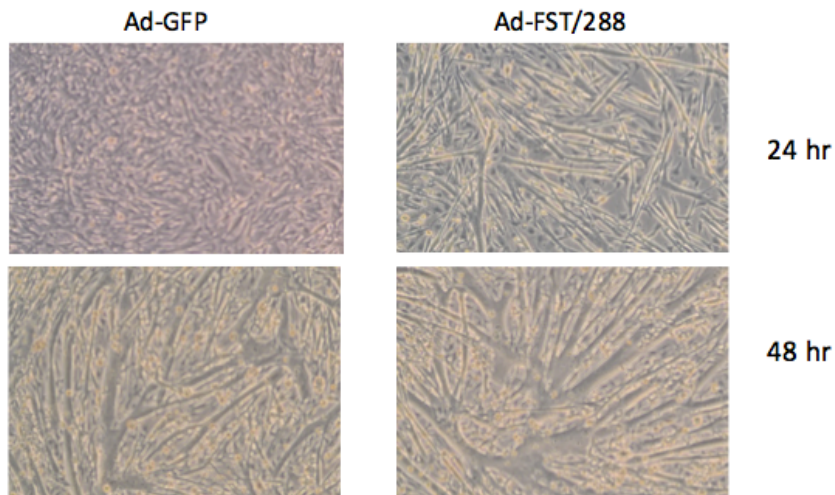
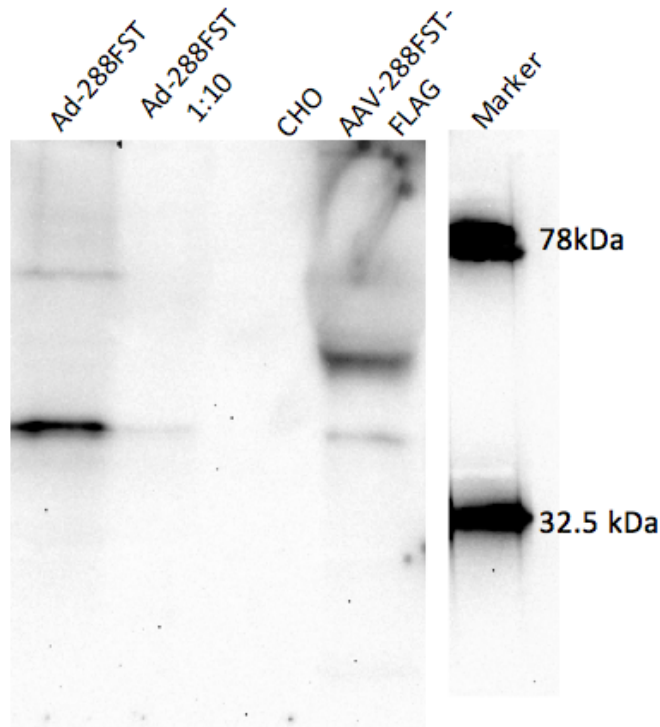


Figure 1: FST-288 stimulates C2C12 mouse muscle differentiation in culture. C2 C12 cells were induced to differentiate using DMEM supplemented with 1% horse serum and insulin. Control Ad-GFP and Ad-FST-288 were added simultaneously with differentiation medium. Ad-FST-288 stimulated precocious differentiation at 24 hr and promoted more extensive differentiation at 48hr in comparison to controls.

- AAV and AV with Recombinant FS-288 administered to Chinese Hamster Ovarian (CHO) cells in vitro produced Follistatin 288 as demonstrated by immunoassay (Figure 2).



**Figure 2.** Immunoblot analysis of recombinant FST-288 protein secreted by suspension-adapted CHO cells 48hr following Ad-FST-288 and AAV-FST-FLAG transduction. Adenoviral-mediated expression of FST-288 appears to be more robust than AAV-FST-288. Blots were probed with anti-FST antibody.

- Bacterial expression of FS-288 protein failed
  - ExpiCHO cell production of FS-288 successful (see Figure 2) but in very low yields
- In vivo, pilot study completed using AV with FS-288 injected into gastrocnemius muscle of rodent
  - After 4 weeks no hypertrophy noted, no FS-288 detected in muscle per immunoassay
- Alternate vendor (Vector BioLabs) identified and commissioned to produce Recombinant DNA FS-288 in Adeno-associated virus (AAV)
  - Though AV seemed to show robust response in vivo, lack of in vitro response indicated (as per our original experimental plan) AAV is appropriate vector (typical vector reported in scientific literature)

- AAV with Recombinant DNA FS-288 delivered and pilot study underway (though results pending approximately 4 weeks)
- Alternate vendor (BioVision) identified and commissioned to produce Recombinant Follistatin 288 protein
  - Protein stability testing already performed by BioVision and protein felt to be stable
  - FS-288 protein received and delivered into pilot study rodents; muscle harvest and testing underway with preliminary data on 2/5 rodents (to be completed over the next week); *see appendix 1*

*Training/Professional development*

Nothing to report

*Result dissemination*

Nothing to report

*Plans during next reporting period*

The pilot study is underway with results of protein treatment due this week (preliminary results look very promising) and results of viral vector wing of study due in 4-5 weeks. If positive response in either wing, we will proceed with formal experimental protocol as planned.

If either wing has a negative result possible courses of action would include:

- Repeating pilot study with FS-315 isoform
- Abandoning that wing of the study
- Proceed with limited injury model study to assess differences in protein expression/response in normal and abnormal muscle (it is possible that FS-288 causes physiological response only in healing or growing muscle)
- All possibilities would require discussion with scientific officer to determine most appropriate course of action

#### **4. Impact**

*On principle discipline:*

Determination that AV is not a useful delivery tool for Recombinant FS-288 to muscle cells.

*On other disciplines:*

Nothing to report

*On technology transfer:*

Nothing to report

*On society:*

Nothing to report



## 5. Changes/Problems

### *Changes in approach:*

- The decision was made to switch viral vector for delivery of Recombinant FS-288 from Adenoassociated Virus (AAV) as per our original experimental plan, to Adeno Virus (AV) based on recommendations from our Biological Macromolecular core facility. Their recommendation was based on inability to produce AAV in sufficient quantities, perceived easier (and more economical) production of AV, and a history of successful use of AV for protein production. After negative pilot study using the AV, immunoassay testing revealed a complete lack of FS-288 protein in treated muscle indicating delivery failure. AAV was the typical vector used in prior studies to deliver FS-288 so we reverted to our original plan. We identified several alternate sources (vendors) for production of DNA FS-288 packaged in AAV. Since the core facility was unable to deliver the AAV, we ended our relationship with them and commissioned production, purification, and delivery of Recombinant Follistatin DNA FS-288 packaged in AAV with a commercial vendor (Vector BioLabs).
  - Because of the initial failed pilot study, five more animals were requested to perform pilot testing on AAV
- Despite several months of effort, the VCU Biological Macromolecular core facility was unable to produce adequate amounts of Recombinant Follistatin Protein. A commercial vendor was identified that had the protein in stock. We requested the core facility to cease further efforts and obtained the protein from BioVision.

### *Actual or anticipated problems or delays*

- *Problem:* The VCU Biological Macromolecular core facility took significantly longer than we had budgeted (based on their guidance) to produce testable viral vector packaged Recombinant FS-288 DNA or Recombinant FS-288 protein. Once product was delivered, we immediately initiated planned pilot study. However, the provided vector failed to produce in vivo response. The core facility despite several months delay were never able to produce the FS-288 protein (though we believe that they tried several strategies).  
*Response:* Commercial vendors were identified and have already provided viral vector packaged Recombinant FS-288 DNA and protein
- *Problem:* AV packaged Recombinant FS-288 failed to produce hypertrophy in rodent muscle; no FS-288 protein detected in muscle  
*Response:* AAV will be tested as delivery vector

### *Impact on expenditures*

- Core facility expenditures did not yield results and additional expenditures generated by commercial vendors
  - No additional funding needed to cover this added expense
- Current delays have put us approximately 6 months behind schedule

- Current funding adequate to complete the study though a no cost extension will be necessary.

*Significant changes in use of human subjects*

Nothing to report

*Significant changes in use of vertebrate animals*

Five additional animals needed to expand pilot study based on failure of initial wing. IACUC and ACURO both notified and approved.

*Significant changes in use of biohazards*

The temporary use of AV required a higher level of biological safety than for AAV. This was supervised and approved by VCU OEHS.

## 6. Products

*Publications/presentations*

Nothing to report

*Website/Internet*

Nothing to report

*Technologies*

Nothing to report

*Inventions/patents*

Nothing to report

*Other products*

Nothing to report

## 7. Participants

*Individuals working on project*

Name:	Jonathan Isaacs, M.D.
Project Role:	PI
Researcher Identifier	
Nearest person month worked:	12
Contribution to project:	Regulatory process, supervising pilot study
Funding support:	VCU salary, MCV physicians salary for clinical work, protocol no. ANG-CP-007, Cook Biotech, Inc. industry grant, Flow-through funding from NIH: 1R34NS097113-01

Name:	Satya Mallu, M.D.
Project Role:	Co-investigator
Researcher Identifier	
Nearest person month worked:	12
Contribution to project:	Assisted with regulatory process, performed pilot work
Funding support:	VCU salary, Cook Biotech, Inc. industry grant, Flow-through funding from NIH: 1R34NS097113-01, AFSH grant

Name:	Gaurangkumar Patel, B.S.
Project Role:	Lab technician
Researcher Identifier	
Nearest person month worked:	12
Contribution to project:	Pilot study
Funding support:	VCU salary

Name:	Mary Shall, PhD
Project Role:	Co-PI
Researcher Identifier	
Nearest person month worked:	4
Contribution to project:	Pilot study
Funding support:	VCU salary

Name:	Scott Vota, DO
Project Role:	Co-investigator
Researcher Identifier	
Nearest person month worked:	4
Contribution to project:	Pilot study
Funding support:	VCU salary and MCV physicians salary for clinical work

Name:	Jeffery Dupree, PhD
Project Role:	Co-investigator
Researcher Identifier	
Nearest person month worked:	4
Contribution to project:	Pilot study
Funding support:	VCU salary and NIH grants

**8. Special reporting**

*collaborative awards*

Nothing to report

*Quad chart*

Attached

**9. Appendices**

- 1) Preliminary results of protein administration pilot study
- 2) Quad chart

## Follistatin Pilot Report

Below are the results from following animals:

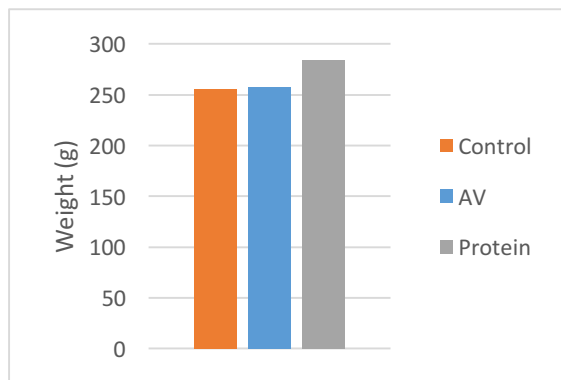
Group	n
AV-288 Follistatin	5
Control	5
Follistatin Protein	2

### Animal Weight (grams):

AV FS-288 (FPP6 to FPP10)	
Average	256.72
STDEV	6.28267459

Control (FPP1 to FPP5)	
Average	255.9
STDEV	6.979254975

FS Protein (FPP11 to FPP15)	
Average	284
STDEV	1.414213562

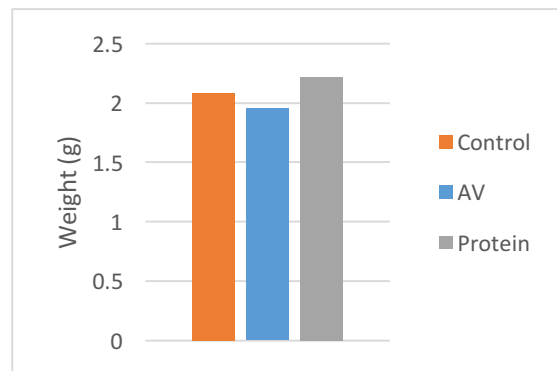


### Muscle Weight (grams):

AV FS-288 (FPP6 to FPP10)		
	EXP	CTRL
Average	1.9548	2.0428
STDEV	0.157862915	0.102416795

Control (FPP1 to FPP5)		
	EXP	CTRL
Average	2.0834	2.1404
STDEV	0.173822611	0.175427478

FS Protein (FPP11 to FPP15)		
	EXP	CTRL
Average	2.215	2.305
STDEV	0.049497475	0.049497475

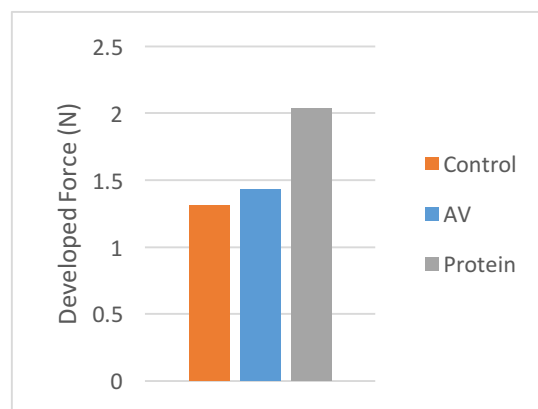


### Developed Force:

AV FS-288 (FPP6 to FPP10) EXP		
	EXP	CTRL
Average	1.4298	1.7034
STDEV	0.357386625	0.437589762

Control (FPP1 to FPP5) EXP		
	EXP	CTRL
Average	1.3118	1.3146
STDEV	0.704691919	0.482312451

FS Protein (FPP11 to FPP15) EXP		
	EXP	CTRL
Average	2.0355	2.5555
STDEV	0.084145707	0.549421969



### Conclusions:

- AV FS-288 Virus had no positive effect on the muscle
- In FS Protein group, increased animal weight was observed when compared to control group by 28 grams on average. This equates to 11% increase in total animal weight.
- In FS Protein group, increased muscle weight was observed when compared to control group by 0.1316 grams (EXP) and 0.1646 grams (CTRL) on average. This equates to increase in muscle weight of 6.3% on EXP side and 7.9% on CTRL side.
- In FS Protein group, increased developed force was observed when compared to control group by 0.7237 N (EXP) and 1.2409 N (CTRL) on average. This equates to increase in developed force of 55.2% on EXP side and 94.4% on CTRL side.
- FS Protein is observed to have more systematic effect on the animal vs. local effect even when it is administered locally. This observation is based on the muscle weight and developed force values.
- FS Protein is observed to induce muscle hypertrophy as seen by the increase in all 3 measured parameters.
- Some of the animals from Follistatin Protein group still under study and will be completed by September 20, 2016.
- No statistical analysis is completed at this time, but will be by September 21, 2016.
- Protein Analysis will be conducted using ELISA on September 23, 2016.

# Follistatin: A Potential Anabolic Treatment for Re-Innervated Muscle

Proposal #11231008



PI: Jonathan Isaacs, MD

Org: Virginia Commonwealth University

Award Amount: \$705,041

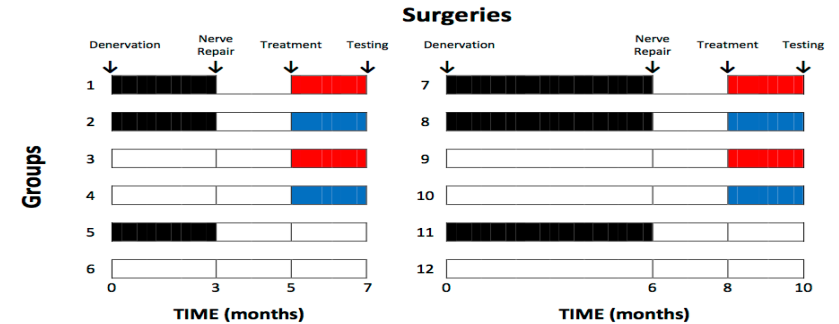
## Study Aims

- To utilize an established animal model of denervation atrophy to determine if Follistatin treatment (administered either as a recombinant protein or as a recombinant DNA) will improve muscle recovery following re-innervation after prolonged periods of denervation.
- To determine Follistatin effects on nerve regeneration and intramuscular fibrosis (in re-innervated tissue).

## Approach

Based on the pilot study result, rodents will undergo transection of one tibial nerve to denervate the hind limb muscles (including gastrocnemius). After a delay (of either 3 or 6 months) the nerve will be repaired and the muscles re-innervated. The re-innervated muscle will be treated with either recombinant follistatin protein (delivered thru an implantable drug delivery system) or recombinant follistatin DNA (delivered thru adeno viral vectors injected into the reinnervated gastrocnemius muscle). After 8 weeks recovery, the effects of the follistatin treatment will be determined utilizing strength testing, muscle morphology, muscle histology, and muscle immunohistology (to determine muscle fiber type distribution and satellite, or regenerative cell, population pools). Nerve conduction testing will be performed to differentiate follistatin effects on nerve regeneration and function; muscle staining for collagen will determine effects on muscle fibrosis; and follistatin levels will be measured in treated muscle to confirm effective dosing and delivery of follistatin. Test results will be compared with sham surgery (plus FS treatment), re-innervation (without treatment), and control groups.

## Follistatin Project Experiment Timeline



1. Recombinant Follistatin Protein (FS-288)	EXP	7. Recombinant Follistatin Protein (FS-288)
2. Recombinant Follistatin DNA (FS-288)	EXP	8. Recombinant Follistatin DNA (FS-288)
3. Recombinant Follistatin Protein (FS-288)	SHAM SURGERY	9. Recombinant Follistatin Protein (FS-288)
4. Recombinant Follistatin DNA (FS-288)	SHAM SURGERY	10. Recombinant Follistatin DNA (FS-288)
5. Only Carrier (Buffer Saline)	SHAM TREATMENT	11. Only Carrier (Buffer Saline)
6. Only Carrier (Buffer Saline)	CTRL	12. Only Carrier (Buffer Saline)

Note: EXP and SHAM TREATMENT undergo DENERVATION Surgeries; SHAM SURGERY and CTRL have "NO" DENERVATION Surgeries.

## Timeline and Cost

Activities	CY	15	16	17	18
Regulatory Process & Pilot Project					
Denervation and re-Innervation of hind limb muscles, Treatment with FS and Testing					
Histology, Manuscript Preparation and Presentation					
Estimated Budget (\$)		\$10762	\$390,356	\$303,923	

## Goals/Milestones

**CY15 Goal – Utilize an established rodent model of denervation atrophy**

✓ Regulatory Process – Received ACURO approved on Jan 19, 2016

**CY16 Goals – Treat re-innervated muscle with Follistatin, Determine treatment effects utilizing strength testing, muscle morphology, electrophysiology nerve testing**

✓ Pilot Project Started

- Denervation of hind limb muscles
- Reinnervation of hind limb muscles
- Synthesis of Recombinant Follistatin DNA/Protein
- Treatment with Follistatin
- Testing of muscle recovery/nerve regeneration
- Immunohistology staining and histology of muscle
- Measurement of Follistatin levels in muscle

**CY17 Goal – Histology (of nerve and muscle), Manuscript preparation, Presentation**

- Histology of muscle/nerve
- Data Analysis
- Manuscript Preparation

## Comments/Challenges/Issues/Concerns

- Pilot Project is added after consulting with GOR. Pilot project started with Follistatin DNA (with Adeno Virus) and Control Groups. FS-DNA with AV did not show any Follistatin Protein in muscle. We are working on Follistatin DNA group with Adeno Associated Virus (AAV) vector delivery.

## Budget Expenditure to Date

Projected Expenditure: \$705,041

Actual Expenditure: \$194510

Updated: Richmond, VA. Sep 14, 2016